Rapid detection of *Haemophilus ducreyi* in clinical and experimental infections using monoclonal antibody: a preliminary evaluation

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SUMMARY A monoclonal antibody raised against *Haemophilus ducreyi* was tested for its sensitivity and specificity as an immunofluorescence (IF) reagent using simulated vaginal smears containing *H. ducreyi*, smears taken from skin lesions of mice infected with *H. ducreyi* and patients from South Africa, Thailand and Malaysia with clinically diagnosed chancroid. The IF test was more sensitive than culture or Gram staining in the simulated smears, theoretically detecting <4 organisms/sample. It detected *H. ducreyi* in 95% of the animal lesions compared with 14% detected by culture. Immunofluorescence testing identified over 90% of culture-positive cases of chancroid but also detected organisms in some culture-negative cases where clinical evidence for the diagnosis was strong. These results suggest that this antibody may provide a simple, rapid and sensitive means of detecting *H. ducreyi* in cases of chancroid.

Chancroid, the most common cause of genital ulcer disease in many developing countries,¹⁻⁴ has recently become re-established as a significant sexually transmitted disease in the United States⁵ and outbreaks have sporadically occurred in other temperate regions.^{6,7} In many areas where the disease is endemic, chancroid remains a purely clinical diagnosis because Gram stained smears made from the exudate obtained from ulcerations are unreliable⁸ and isolation of the causative bacterium, *H. ducreyi*, is difficult because the organisms are fastidious and the media required for primary isolation expensive. By using a single enriched selective medium and optimal cultural conditions the isolation rate of *H. ducreyi* from presumptive chancroidal ulcerations has been estimated to be 60-70%, with higher rates being achieved if two media are employed.⁹ Clearly there remains a need for a simple, specific and sensitive test for the diagnosis of the

disease. Similar difficulties have been encountered in establishing a diagnosis in two other sexually transmitted diseases, namely, chlamydial infection and genital herpes. Demonstration of these organisms by non-cultural methods has been achieved by the development of monoclonal antibodies to *Chlamydia trachomatis* and HSV-2, and their subsequent use in the detection of specific antigens in clinical smears.^{10,11}

Monoclonal antibodies to *H. ducreyi* have previously been used to detect these organisms in ulcer material obtained from experimental animals using a radio-immunoblotting technique¹² and in a limited number of clinical cases using immunofluorescence.¹³ We have raised similar monoclonal antibodies to *H. ducreyi* which react with a single polypeptide band (MW 29,000 kd), in the outer membrane protein of the bacterium (Finn *et al. J Med Microbiol.* In press).

In this communication we further describe the use of one of these monoclonal antibodies in the diagnosis of the disease, by comparing the results of immunofluorescence staining with those obtained using culture. Both techniques have been applied to simulated vaginal smears prepared in the laboratory

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and to specimens obtained from an animal model of chancroid infection. In addition we have performed a preliminary evaluation of the immunofluorescence technique on clinical material obtained from genital lesions in an area where chancroid is thought to be common but where facilities for culture of *H. ducreyi* are not available and also from *H. ducreyi* culture-positive and culture-negative cases of genital ulcer disease and fluctuant lymphadenopathy from two different geographic regions where routine culture for *H. ducreyi* is performed.

Materials and methods

Bacterial strain

H. ducreyi strain 3138, originally isolated from a case of chancroid in Kenya was kindly provided by Professor P Piot, Institute of Tropical Medicine, Antwerp, Belgium. This strain was used as the immunogen in the production of monoclonal antibodies and was maintained on medium comprising Mueller-Hinton agar base (BBL), 5% chocolate horse blood (Oxoid), 5% foetal calf serum (Gibco), 2% IsoVitaleX (BBL) and 3 mg/l vancomycin in an atmosphere containing 10% CO₂ at high humidity for 48 hours.

Immunisation of mice and production of monoclonal antibodies

Eight week old Balb/C mice were bled immediately prior to being immunised subcutaneously with 0.2 ml of 10⁷ cfu/ml of *H. ducreyi* strain 3138 suspended in Freund's incomplete adjuvant. They were injected with a further 0.2 ml of antigen in saline intraperitoneally on day 28, and intravenously on day 31. Three days later the mice were killed, their spleens removed and spleen cell suspensions prepared and fused with NSO myeloma cells using the technique of Kohler and Milstein.¹³ The supernatants of the resulting hybrid cultures were screened for antibody using indirect immunofluorescence and a dot-blot technique. Antibody-producing hybridomas were isolated and cloned by passage through five limiting dilutions. Three cell lines showing highly reactive antibody were expanded. The antibodies were purified through protein-A-sepharose columns (Sigma), characterised and stored in aliquots at -70°C.

Indirect immunofluorescence staining of *H. ducreyi* using monoclonal antibody

Twenty microlitres of a 1 in 200 dilution of the monoclonal antibody was layered on to appropriate specimens. The slides were incubated for 30 minutes at 37°C in a humid atmosphere, washed twice in PBS (pH 7.3), once in distilled water, and then air dried. They were then stained with fluorescein-conjugated rabbit anti-mouse immunoglobulin (Dakopatts) for a further

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30 minutes at 37°C in a humid atmosphere. The conjugate was used at a dilution of 1 in 35 as determined by previous titration. The slides were finally washed in PBS (pH 7.3), counterstained in 0.1% Evans Blue for 5 minutes, washed in distilled water and air dried. Slides were examined under the oil immersion lens of a fluorescence microscope at × 1000 magnification and specific fluorescence (defined as clumps of, and/or single bacilli showing apple-green fluorescence) sought.

Preparation of simulated smears

Overnight cultures of *Neisseria gonorrhoeae*, *Gardnerella vaginalis*, *Haemophilus influenzae* and *Haemophilus parainfluenzae* grown on solid media were harvested into sterile PBS (pH 7.3) and mixed with vaginal washings obtained following the instillation of sterile PBS (pH 7.3) into the vagina of a woman with no evidence of genital infection. This suspension containing vaginal epithelial cells and approximately 10⁷ mixed bacteria per ml was subsequently used as a negative control and used to make serial 10-fold dilutions of *H. ducreyi* obtained from solid media. Smears were made from a 20 µl aliquot of each dilution and stained by Gram's method or air-dried, fixed in 100% methyl alcohol for 10 minutes and stained by the immunofluorescence technique using monoclonal antibodies. In addition, each suspension was quantitatively cultured for *H. ducreyi* using the Miles-Misra technique¹⁴ on Mueller-Hinton-based medium as described above.

Animal model for chancroid

A murine model of chancroid recently developed at the Clinical Research Centre (CRC), Northwick Park Hospital, Harrow, Middlesex¹⁵ was used to assess the value of the indirect immunofluorescence test using monoclonal antibody. Approximately 10⁷ cfu of *H. ducreyi* strains isolated from cases of clinical chancroid in South Africa and two reference strains (IP 54-2 and 35000) were administered intradermally into the flanks of 8-week old CBA mice. Following development of lesions at the injection site, swabs were taken from the ulcers daily. Smears of the ulcer exudate were made on glass slides and fixed in 70% methanol for 10 minutes. The slides were washed twice in PBS, once in distilled water, dried in air and stored at -70°C. Swabs were also plated directly on to Columbia agar (BBL) supplemented with 10% sheep blood (Wellcome), 2.5% heat inactivated foetal calf serum (Flow Laboratories), 1.5% IsoVitaleX (BBL) and 3 mg/l vancomycin (Eli Lilly) for isolation of *H. ducreyi*.

Clinical smears

Material was obtained from the bases of clinical

lesions and infected lymph nodes of patients with genital ulcer disease, seen in Johannesburg, South Africa, Bangkok, Thailand, and Kuala Lumpur, Malaysia. Cultures for *H. ducreyi* were performed only in Johannesburg and Bangkok. In Johannesburg swabs from primary ulcers were inoculated on to two media in attempts to isolate *H. ducreyi*. The first consisted of Mueller-Hinton agar base (BBL) with 5% chocolate horse blood, 1% IsoVitaleX, and 3 mg/l vancomycin. The second contained gonococcal agar base (Gibco) 2% bovine haemoglobin, 5% foetal calf serum, (Flow Laboratories) 1% IsovitaleX and 3 mg/l vancomycin.

In Bangkok, material aspirated from fluctuant lymph glands was plated on to medium comprising Columbia agar base (Oxoid), 5% foetal calf serum, (Flow Laboratories), 5% rabbit blood and 3 mg/l vancomycin. Inoculated plates were incubated at 35°C in Johannesburg and 34°C in Bangkok in an atmosphere containing 5% CO₂ in air, and examined daily for bacterial growth. In both centres, isolates were identified as *H. ducreyi* by colonial morphology, Gram-staining characteristics and biochemical criteria as described by Kilian.¹⁶ In each case, smears were made from material collected from infected sites, air-dried, fixed in methyl alcohol or acetone for 10 minutes and sent to London where indirect immunofluorescence staining was performed.

Results

Simulated smears

The relative sensitivities for of Gram staining, culture and immunofluorescence using monoclonal antibodies for the detection of *H. ducreyi* in simulated smears are shown the Table.

Typical clumps of Gram-negative rods were not detected in Gram stained smears containing less than 20 cfu *H. ducreyi*. Culture was found to be more sensitive, but still required at least 4 cfu for reproducible detection of *H. ducreyi*. In comparison, the immunofluorescence test using monoclonal antibodies was capable of detecting *H. ducreyi* at counts as low as

one cfu. However, at dilutions containing less than 4 bacteria per 20 µl sample, several high power fields had to be scanned in order to detect a single fluorescing bacterium.

Murine model for chancroid

Overall, 238 swabs obtained from experimental murine ulcers were cultured for *H. ducreyi*. Of these, bacteria were reisolated in 34 cases (14%). In contrast, 69 of 73 specimens (95%) taken for detection of *H. ducreyi* by immunofluorescence were positive. *H. ducreyi* was detected by immunofluorescence up to 15 days following inoculation, but the organisms were rarely recultured from lesions after eight days.

Clinical specimens

Of 34 smears obtained from unselected genital ulcers seen in Kuala Lumpur, Malaysia, 19 (56%) were positive by immunofluorescence. A total of 23 specimens obtained from primary genital ulcerations seen in Johannesburg were cultured for *H. ducreyi* and smears stained with monoclonal antibody. Fifteen were found to be *H. ducreyi*-positive by culture of which 14 were also positive by immunofluorescence. Of these eight specimens which were *H. ducreyi* culture-negative, three were positive by immunofluorescence and five were negative.

Of 34 specimens of bubo material obtained in Bangkok, nine were found to be *H. ducreyi*-positive by culture (26%). Of these, eight were also positive by immunofluorescence. Of those 25 specimens found to be *H. ducreyi* culture-negative, 11 were positive by immunofluorescence. Using culture as the denominator, immunofluorescence had a sensitivity of 93%, a specificity of 63%, a positive predictive value (PPV) of 82% and a negative predictive value (NPV) of 83% for primary ulcerations seen in Johannesburg. The test also had a sensitivity of 89%, a specificity of 56%, a PPV of 42% and NPV of 93% for the bubo study undertaken in Bangkok.

However, this presupposes that culture is in fact the "gold standard" for the diagnosis of chancroid. In this study we have presented considerable evidence that

Comparison of Gram staining, culture and immunofluorescence staining in the detection of *H. ducreyi* in simulated vaginal smears

Theoretical count of <i>H. ducreyi</i> per 20 µl sample	Tests used to detect <i>H. ducreyi</i>		
	Gram staining	Culture	Immunofluorescence staining
20,000	+	+	+
2,000	+	+	+
200	+	+	+
20	+	+	+
4	—	+	+
1	—	—	+

+ = positive; — = negative

the immunofluorescence test can detect *H. ducreyi* in culture-negative cases of chancroid and is in fact more sensitive.

Discussion

At present, despite a recognised lack of sensitivity, culture of *H. ducreyi* is considered the method of choice for the diagnosis of chancroid and has largely superseded the use of Gram stained smears, intra-dermal skin tests and the establishment of a diagnosis on purely clinical grounds. However, it is generally accepted that even under ideal conditions when two selective media are used, culture has a sensitivity of only 80%.⁹ This lack of sensitivity of the existing culture techniques highlights the need for a simple, sensitive, non-culture test which could be employed to establish a definitive diagnosis in individual cases of genital ulcer disease.

The results obtained in this study indicate that since fewer negative results were obtained by immunofluorescence than by culture, the monoclonal antibody technique is either more sensitive than isolation of *H. ducreyi* or has a high rate of false positivity. The results of preliminary experiments on simulated clinical specimens clearly indicate that the immunofluorescence technique is far more sensitive than culture to the extent that the test is theoretically capable of detecting a single bacterium, but that culture-positivity can only be assured when approximately four bacteria are present in the inoculum.

Further evidence to suggest that immunofluorescence is more sensitive than culture was obtained by the use of the murine model. Thus, only 34 of 238 specimens (14%) were positive by culture compared with 69 of 73 (95%) which were positive by immunofluorescence. These findings would appear to indicate that the vast majority of bacteria detected after intradermal inoculation of *H. ducreyi* in mice were non-viable and that the immunofluorescence test is capable of detecting both viable and non-viable bacteria. Indeed, heat-killed *H. ducreyi* inoculated into mouse skin has subsequently been detected by the immunofluorescence technique (M Tuffrey, CRC, Harrow, Middlesex, personal communication).

In the clinical studies reported here, many more positives were obtained using immunofluorescence than by culture, a finding which is consistent with the findings of the laboratory studies. Thus if all cases which were immunofluorescence-positive in the clinical study were in fact chancroid, the sensitivity of the isolation technique employed could be accurately assessed as 82% for ulcers and 42% for bubos. These figures are in agreement with previous studies where investigators have failed to isolate *H. ducreyi* from approximately 19% of primary lesions which were

clinically diagnosed as chancroid and from a higher proportion of bubos.⁹ Unfortunately in neither of the series was a complete range of diagnostic tests undertaken to exclude other aetiologies and thus to determine the absolute specificity of the immunofluorescence test. A comprehensive microbiological study employing microscopic, culture and serological techniques to exclude all possible differential diagnoses and thus determine accurately the specificity of the test is currently in progress.

Unfortunately, the subjective nature of immunofluorescence tests and the need for an expensive immunofluorescence microscope preclude the use of the test in many peripheral clinics where chancroid is endemic, but tests could easily be performed at regional reference centres. However, in the absence of culture facilities, the test can be used to determine whether chancroid is a significant cause of genital ulcer disease in developing societies (prevalence studies) and could also prove a valuable research tool in studies on the pathogenesis and epidemiology of the disease. Even in this limited evaluation, the results obtained from the Malaysian specimens indicate that chancroid is a significant cause of genital ulcer disease in that country. This conclusion was reached despite the specimens being sent to London by mail and arriving three months after collection. Meanwhile, it is clear that further development of diagnostic tests using monoclonal antibodies should be investigated, such as ELISA and immunoblotting.

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